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# II 型单纯疱疹病毒糖蛋白 D 的晶体结构解析

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**摘　要:** II 型单纯疱疹病毒 (HSV-2) 糖蛋白 D (Glycoprotein D，gD) 在介导该病毒入侵到宿主细胞中起着关键作用。为了更好地研究 gD 在病毒侵入过程中的作用机制，利用杆状病毒表达系统表达了 gD 胞外部分区域 (1~285 aa)，通过 Ni-NTA 亲和层析以及分子排阻层析纯化后，得到的带 His 标签的分泌型可溶蛋白，用悬滴法对该蛋白进行了晶体筛选，获得了高质量的晶体。晶体生长条件为 0.1 mol/L Hepes 缓冲液 (pH 7.2)，5% (*V*/*V*) 2-甲基-2,4-戊二醇 (MPD)，10% PEG10000，18 ℃。晶体能够衍射到 1.8 Å，属于 P21 空间群，晶胞参数为 *a*=63.6，*b*=55.4，*c*=65.3 Å，*β*=96.3°。该晶体的获得将为进一步研究 gD 的结构提供了基础，也为 gD 等同类囊膜蛋白的表达、纯化及结构研究提供了重要的参考。

**关键词:** 杆状病毒表达系统，纯化，II 型单纯疱疹病毒，糖蛋白 D 结晶

# X-ray diffraction analysis of glycoprotein D from Herpes simplex virus type 2

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**Abstract:** Glycoprotein D (gD) of Herpes simplex virus type 2 (HSV-2) is a key factor mediating the entry of HSV-2 into host cells. In order to explain the mechanism underlying the gD-mediated receptor-binding and viral entry, we performed a structural study on HSV-2 gD. The ectodomain of the gD protein encompassing residues 1 to 285 was expressed by baculovirus-infected insect cells as a secreted soluble protein with a C-terminal hexa-his tag. The protein was then purified by affinity and size-exclusion chromatography. The purified protein was successfully crystallized using the hanging-drop vapor-diffusion at 18 °C in a condition consisting of 0.1 mol/L Hepes pH 7.2, 5% (*V*/*V*) 2-methyl-2,4-pentanediol (MPD) and 10% PEG 10 000. The crystals diffracted to 1.8 Å resolution and belonged to space group P21, with unit-cell parameters *a*=63.6, *b*=55.4, *c*=65.3 Å, *β*=96.3°.

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**Keywords:** expression by baculovirus-infected insect cells, purification, herpes simplex virus type 2, crystallization of glycoprotein D

## Introduction

Herpes simplex virus (HSV) is a type of enveloped virus which could infect humans of all ages. The virus occurs worldwide and can cause a wide variety of diseases, including gingivostomatitis, keratoconjunctivitis, encephalitis, herpes genitalis and other clinical manifestations. The initial infection by HSV mainly occurs on epithelial tissues, after which the virus could spread to neurons of the peripheral nervous system for latent infections. Based on the antigenicity of the viral surface proteins, herpes simplex virus could be divided into two subtypes: herpes simplex virus type 1 and type 2 (HSV-1 and -2), respectively[1]. The two subtypes also exhibit different tissue tropisms. HSV-1 typically infects oral epithelial tissues, whereas HSV-2 primarily infects the genital mucosa and mainly causes genital lesions[2]. HSV-2 infection can also induce cervical carcinoma and increase the ratio of HIV infection by facilitating the entry of human immunodeficiency virus type 1 into primary macrophages[3-4]. In developing countries, such as China, HSV-2 and HIV co-infection was reported to increase the infectious rate of HIV by four times[5-6]. In addition, HSV-2 is one of the most common causes of neonatal herpes and can also lead to encephalitis in those immune-compromised individuals[7]. By far, several drugs have been shown to be effective in HSV treatment. These drugs, such as acyclovir and valaciclovir, could reduce the release of HSV virion, and thereby cure the patients[8]. Nevertheless, thus far, no drugs or effective treatment methods are available to prevent primary and recurring HSV infections. Therefore, new drugs targeting vial entry process is currently urgent needed. A picture of detailed interactions between the enveloped membrane glycoproteins of HSV and their receptors on host cells is necessary.

Like other enveloped viruses, HSV entry into target cells requires fusion of the viral envelop with the lipid membrane of the cell. Cooperative interactions between various glycoproteins present on the envelope of HSV (including gC, gD, gB, gH and gL) and the host molecules are reportedly involved in this entry process[9]. The initial virus-cell attachment was realized by gC and/or gB interacting with cell surface heparan sulfate (HS) proteoglycans[10]. Then glycoprotein D (gD) binds to a cell surface receptor such as herpes virus entry mediator (HVEM), a TNF receptor family member, or nectin-1, a member of the immunoglobulin superfamily[11-12]. This latter event is followed by membrane fusion mediated by gB and the heterodimer gH/gL[9]. Therefore, binding of gD to one of its functional cellular receptors is of vital importance in the colonization of HSV on the surface of target cells. Apart from its role in the virus-cell attachment, the gD-receptor interaction might also participates in the membrane-fusion step since former reports have demonstrated that the soluble gD ectodomain is able to mediate entry of an HSV virion lacking gD on its envelope (gD-null virus)[13], and the C-terminal profusion domain of gD could form distinct complexes with gB and gH/gL[14-15].

Apart from its important roles in mediating virus entry into host cells, HSV gD protein is also involved in the budding process of progeny viruses[16-17]. Recent studies have shown that egress of HSV from host cells is a highly complexed event involving extensive modification of cellular membranes and sequential envelopment and de-envelopment steps. After the assembly of capsids in the nucleus, primary envelopment at the inner nuclear membrane (INM) occurs, producing enveloped virus particles. Then these particles undergo de-envelopment by fusing with the outer nuclear membrane (ONM), delivering the capsids into the cytoplasm, where a secondary envelopment of the viral capsids occurs. Glycoprotein D participates in the secondary envelopment process, and is possibly also involved in de-envelopment[18-19].

Moreover, gD is also a target of the humoral and cellular immune response of the host. Many monoclonal antibodies of high virus-neutralizing titers are specific for gD protein[20-21], indicating good prospects of this glycoprotein being used in future vaccine development. These unique characters of this virus-oriented protein make it of great interests to explore its structural features at the atomic level.

To date, the crystal structures of a truncated

HSV-1(strain: KOS) gD protein (residues 1-285) alone and in complex with the ectodomain of its cellular receptor HVEM have been reported[22]. The structures reveal a V-like immunoglobulin (Ig) domain at the core of gD, flanked by flexible N- and C-terminal extensions. The same group also reported the structure of a longer form of gD protein consisting of residues 23 to 306[23]. The mystery of HSV-1-gD/receptor interactions are now being unveiled with the assistance of these structures, the structure of HSV-2 gD remains elusive.

Overall, gD proteins from HSV-1 and HSV-2 are common in their roles facilitating viral cell entry. Both proteins could recognize the same cellular receptors including HVEM and nectin-1[24-25]. Nevertheless, the different tissue tropism of HSV-1 and HSV-2 indicates that the two viral gDs might also use different host molecules to mediate cell entry. In support of this, former reports have demonstrated that nectin-2, another member of the immunoglobulin superfamily, could enable the viral entry of HSV-2, but not of wild-type HSV-1[26]. These observations indicate HSV-2 gD should differ from its HSV-1 counterpart at some aspects, determining its specific affinity for molecules like nectin-2. Therefore, a high resolution structure of the gD protein from HSV-2 is necessary in understanding its unique characters.

In this study, we reported the successful expression, purification and crystallization of a truncated form of glycoprotein D (including residues 1-285) from HSV-2 strain 333. A diffraction dataset up to 1.8 Å resolution was collected from the protein crystals. The preliminary crystallographic analysis and the initial structure-solving trials were also reported.

## 1 Materials and methods

### 1.1 Cloning and expression

The truncated HSV-2 gD (GenBank Accession No. EU018091) containing residues 1-285 was designed to be expressed as a secreted protein using the baculovirus expression system. Therefore a gp67 signal peptide and a hexa-His tag were added to the N- and C-termini of the gD protein. The fragment encoding gp67 signal peptide was achieved by PCR using primer pairs GP67-F and GP67-R, and the pAcGP67-B plasmid as the template. The truncated gD (tr-gD) coding sequence was amplified with primers tr-gD-F and tr-gD-R. This latter primer also includes the sequence coding for the hexa-His tag and a stop codon. With the above-obtained DNA segments being mixed in equal molar as the template, the final fragment encoding the recombinant protein was achieved by a further PCR reaction using the GP67-F and tr-gD-R primers. This fragment was then introduced into pFastBac-1 vector via *Eco*R I and *Xho* I restriction sites. The sequencing-verified recombinant pFastBac-1 plasmid was transformed into DH10Bac™ *E. coli* competent cells, and the resultant colonies were analyzed by PCR to select those containing the recombinant bacmid. Subsequently, the bacmid DNA was extracted and transfected into insect Sf9 cells with Cellfectin II Reagent to generate the recombinant baculovirus particles. This original viral stock is low in titer and is therefore used to infect Sf9 cells to produce high-titer viral stocks.

For protein expression, the high-titer viral stock was inoculated into High Five™ cells with a *V/V* ratio of 1:10. The cells were then incubated at 27 °C for 48 h to maximize the amount of the tr-gD protein secreted into the cell culture.

**Table 1 Primers used in this study**

| Primer name | Primer sequence (5′−3′) |
|---|---|
| GP67-F | CGGAATTCATGCTACTAGTAAATCAG |
| GP67-R | GGTCTGCTAAGGCGTATTTAAGATCCGCCGCAAAGGC |
| tr-gD-F | GCCTTTGCGGCGGATCTTAAATACGCCTTAGCAGACC |
| tr-gD-R | CCGCTCGAGCTAATGATGATGATGATGATGCGTCCCGGCGGGATC |

### 1.2 Western blotting assay

A Western blotting assay was used to determine the yield of tr-gD protein using High Five™ cell-line. After the inoculation of the viral stock into High Five™ cells, small aliquots of the cell culture were sampled (24 h, 36 h, 48 h, 60 h and 72 h post-infection) and loaded on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) for separation. The proteins were then transferred to nitrocellulose membrane. The tr-gD protein was detected by an anti-His monoclonal antibody using an enhanced chemiluminescence system (Pierce).

### 1.3 Purification

48 h post-infection, the cell debris was removed by centrifugation, and the subsequent cell culture containing HSV-2 tr-gD was passed through Ni-NTA column (GE Healthcare) at 4 °C with an appropriate flowrate overnight. The column was then washed with 10−15 bed volumes of wash buffer consisting of 20 mmol/L Tris-HCl (pH 8.0), 50 mmol/L NaCl and 20 mmol/L imidazole to remove those unspecifically bounded proteins. The tr-gD protein was then eluted with the wash buffer containing 200 mmol/L imidazole. The eluant was subsequently concentrated in an Amicon Ultra-15 filter (Millipore) using a 10 kDa cutoff membrane, and then loaded to a HiLoad 16/60 Superdex 200 column (GE Healthcare) equilibrated with buffer (20 mmol/L Tris-HCl pH 8.0, 50 mmol/L NaCl) in advance for further purification. The fractions of the tr-gD protein were collected, and analyzed by SDS-PAGE and Coomassie staining.

### 1.4 N-terminal sequencing

The protein sample was sent to School of Life Sciences, Peking University for N-terminal sequencing assay.

### 1.5 Determination of the protein purity by HPLC-based assay

The purified tr-gD protein was analyzed by reverse-phase HPLC on a C4 column (4.6 mm× 250 mm) using a 20 min, 0−30% linear gradient of acetonitrile in 0.1% trifluoroacetic acid. The absorbance was monitored at 215 nm. The tr-gD protein was eluted as a single peak with an apparent retention time of 11.3 min. The amount of the protein was determined by calculation of the protein peak using the integration tool as implemented in Empower 2 software (Waters Corporation).

### 1.6 Crystallization

The pooled fractions of purified HSV-2 tr-gD protein were concentrated to 10 g/L by ultrafiltration using a Millipore Amicon 10 kDa centrifugal device. The initial crystal-screening trials were performed with Hampton Kits using the hanging-drop vapor-diffusion method. 1.5 μL of the protein solution was mixed with 1.5 μL reservoir solution and equilibrated against 200 μL reservoir solution at 291 K for up to two months. After optimization, the final crystals were obtained from a condition consisting of 0.1 mol/L Hepes pH 7.2, 5% (*V*/*V*) MPD and 10% PEG10000.

### 1.7 X-ray data collection

The X-ray diffraction data were collected using the synchrotron radiation (λ=0.979 16 Å) at Shanghai Synchrotron Radiation Facility (SSRF-beamline BL17U). A single piece of crystal was separated from the crystal bunch, and mounted in a nylon loop and flash-cooled in a cryostream of liquid $N_2$ gas before data collection. The cryoprotectant is composed of 15% (*V*/*V*) glycerol and 85% reservoir solution. Data were indexed, integrated and scaled using *DENZO* and *SCALEPACK* as implemented in HKL2000[27]. The final statistics on the collected data are given in Table 2.

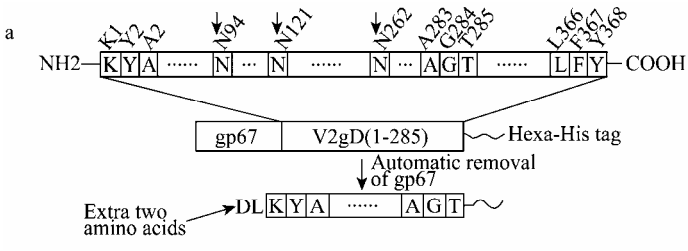
## 2 Results and discussion

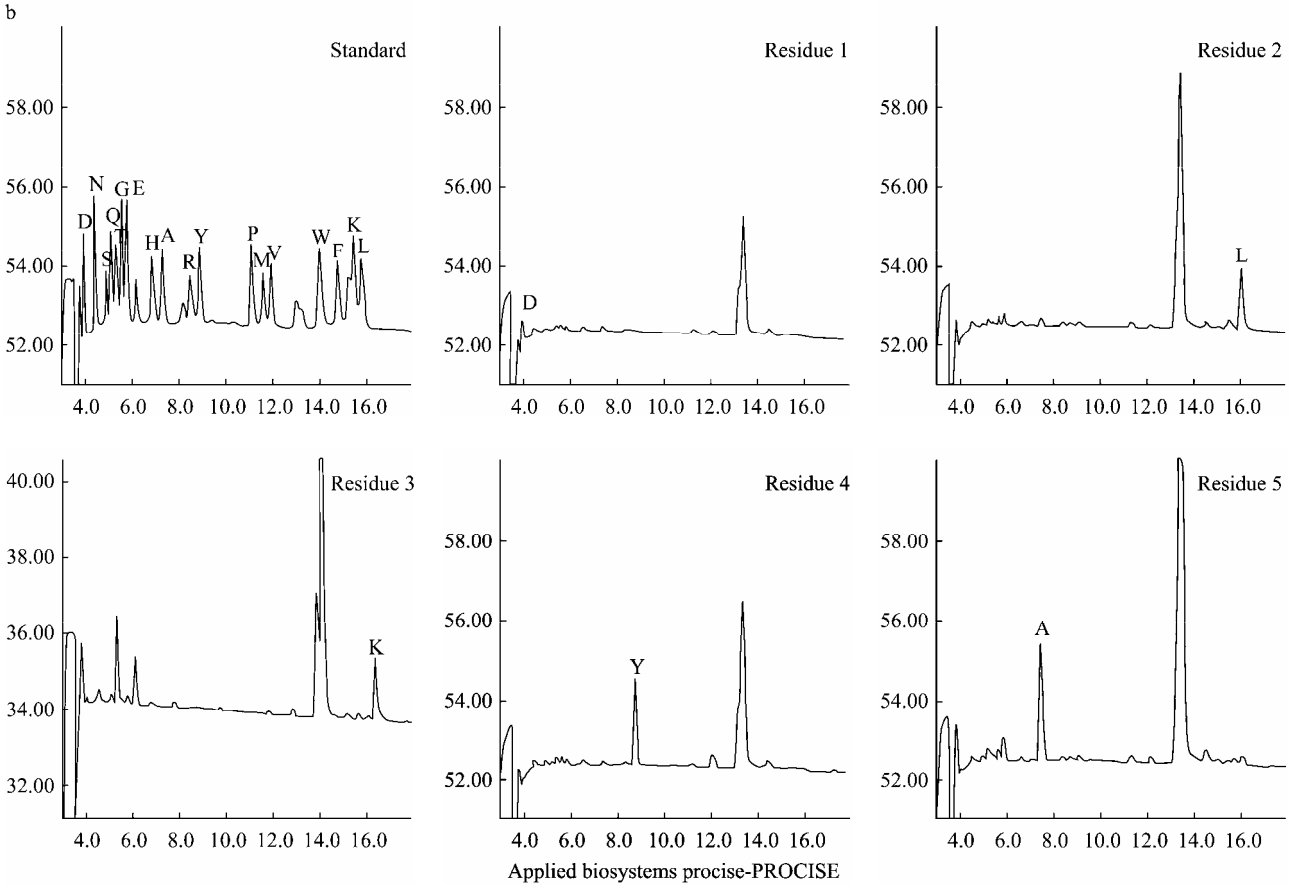
gD of HSV-2 is a 368 amino acid glycoprotein. A truncated form of the protein consisting of residues 1−285 was expressed using the Baculovirus expression system. To secret the protein to the media and to simplify the subsequent purification procedures, a gp67 signal peptide and a His tag were added to the N- and C-termini of HSV-2 gD (Fig. 1a). The removal of the signal peptide was proved to be automatic since the secreted product is about 35 kDa in molecular mass,

**Table 2 Data collection statistics**

| Factor | Data |
|---|---|
| Space group | P21 |
| Cell dimensions | |
| *a*, *b*, *c* (Å) | 63.6, 55.4, 65.3 |
| *α*, *β*, *γ* (°) | 90.0, 96.3, 90.0 |
| Wavelength (Å) | 0.97916 |
| Resolution range (Å) [a] | 50.0−1.8 (1.86−1.80) |
| No. of unique reflections | 40614 |
| $R_{merge}$ [a b] | 0.085 (0.297) |
| Average I/σ (I) [a] | 15.9 (2.71) |
| Completeness (%) [a] | 96.8 (81.2) |
| Redundancy [a] | 3.9 (2.8) |

[a] Values for the outmost resolution shell are given in parentheses. [b] $R_{merge}=\Sigma_i\Sigma_{hkl}|I_i-<I>|/\Sigma_i\Sigma_{hkl}I_i$, where $I_i$ is the observed intensity and <I> is the average intensity from multiple measurements.



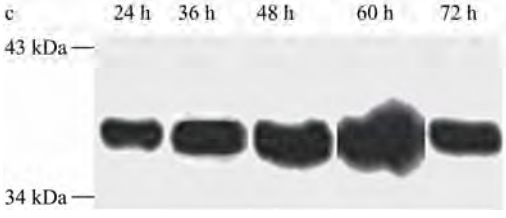




Fig. 1 Expression strategy as well as primary expression trials of HSV-2 tr-gD (1-285) as a secreted soluble protein. (a) Schemes showing the strategy for generation of secreted HSV-2 tr-gD. The residue numbers are defined based on the mature form of gD protein which starts from Lys1 and ends at Tyr368. The three N-linked oligosaccharide sites are indicated by black arrows. A gp67 signal peptide and a hexa-His tag were added to the N- and C-termini of HSV-2 tr-gD (1-285). The gp67 peptide was automatically removed after secretion, and two extra residues remain at the N-terminus of the protein. (b) The protein N-terminal sequencing result showing that the five residues at the N-terminus were Asp, Leu, Lys, Tyr and Ala, respectively. (c) The yield of tr-gD protein at different time points. The standard molecular-weight markers are indicated.

corresponding well to the tr-gD protein plus hexa-His residues and several glycans at the three N-linked oligosaccharide sites (Fig. 1a). After removal of gp67, two extra amino acids were still left at the N-terminus of the tr-gD protein. This was later confirmed by the protein N-terminal sequencing result showing that the five residues at the N-terminus were Asp, Leu, Lys, Tyr and Ala, respectively (Fig. 1b).

The yield of the tr-gD protein using High Five$^{TM}$ cell was also determined at different time points. As shown in Fig.1c, with recombinant baculovirus, the best tr-gD production was observed at 48 h and 60 h post-infection. Despite that the yield of the tr-gD protein is even better at the 60 h time-point, to balance the time consumption and the final yield of the protein, the cell culture was harvested 48 h after the inoculation of the high-titer viral stock into High Five$^{TM}$ cells.

The expression of HSV-2 tr-gD as a secreted soluble protein extremely simplifies the subsequent purification procedures. After one step of purification using the Ni-NTA resins, the purity of the protein could be over 80%. The protein was then further purified by size exclusion chromatography.As shown in Fig. 3a, with an elution volume of 88.6 mL on the superdex200 column, HSV-2 tr-gD behaves as an asymmetric peak in the 280 nm absorbance profile, indicating the high homogeneity of the protein. The final purity of the tr-gD protein was proved to be over 99% on 12% SDS-PAGE (Fig. 2a). We further quantitatively determined the purity of the tr-gD protein using an HPLC based assay. The protein was loaded onto a C4 column and separated using a 20 min, 0−30% linear gradient of acetonitrile in 0.1% trifluoroacetic acid. As shown in Fig.2b, the protein was eluted as a single peak with an apparent retention time of 11.3 min. The peak area was calculated by integration, and the final purity of the protein was over 99%.

The initial crystallization condition for the purified protein was obtained by screening trials using the Hampton Kits. After optimization, fine diffracted crystals were obtained from a condition consisting of 0.1 mol/L Hepes pH 7.2, 5% (*V/V*) MPD and 10% PEG 10 000 by hanging-drop vapour-diffusion method. It takes over two months for the crystals to grow (Fig. 3a). The diffraction data was collected using the synchrotron radiation, and the crystal could diffract to 1.8Å resolution (Fig. 3b). The final data-set statistics are summarized in Table 2. The crystal lattice belongs to Space group P21, with unit-cell parameters $a$=63.6, $b$=55.4, $c$=65.3 Å, $\beta$=96.3°.

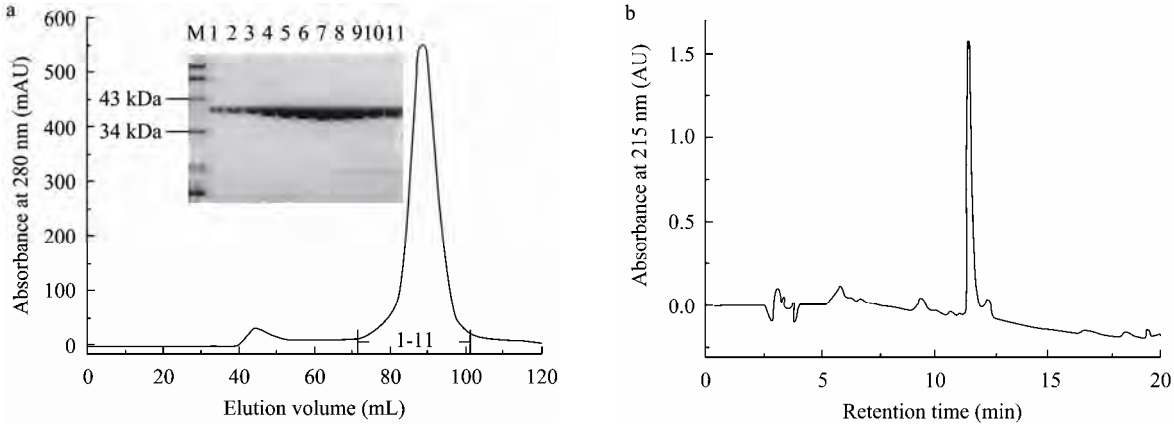



Fig. 2 Purity determination of HSV-2 tr-gD. (a) Purification of the HSV-2 tr-gD protein by gel filtration. A typical separation profile of the tr-gD protein on superdex200 (Hiload 16/60) column was shown. Fractionations of the tr-gD protein were analyzed by 12% SDS-PAGE. Lane *M*: the standard protein molecular-weight markers. (b) The result of the HPLC based assay showing that the final purity of the HSV-2 tr-gD protein was over 99%.

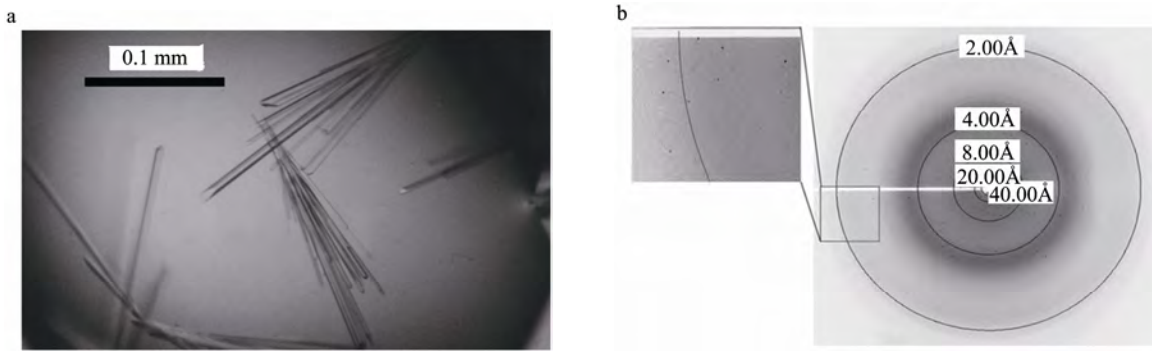



Fig. 3 Crystallographic characterization of HSV-2 tr-gD. (a) Representative crystals of the tr-gD protein from HSV-2. (b) A typical diffraction image collected from a crystal of HSV-2 tr-gD. The resolution shells are shown.

The initial molecular-replacement (MR) calculations were performed using the program AMoRe (CCP4 suite) with the structure of HSV-1 gD (pdb code: 1L2G) as the search model. The gD proteins from the two viruses are of 88% in sequence identity, and the MR trial reveals a possible solution based on which a feasible structural model for HSV-2 tr-gD was built. The structure has recently been refined to 29.6 and 25.2 for $R_{work}$ and $R_{free}$, respectively, and contains two molecules per asymmetric unit. Further refinement of the structure is now underway.

Overall, the full-length gDs from HSV-1 and HSV-2 exhibit an identity of about 84.8% in sequence. This similarity is even higher for the ectodomains of the two glycoproteins, with residues 1-285 of the two gDs sharing over 88% sequence identity. Therefore significant structural similarities between HSV-1 and HSV-2 gDs could be expected. Previous reports have demonstrated that HSV-1 gD is mainly an IgV-like barrel core with N- and C-terminal extensions. According to these structures, those varied amino acids between the gD proteins of the two Herpes simplex virus types are mainly present on the surface of the barrel core and the terminal extensions. This might be the structural basis for the specific binding of HSV-2 gD, but not of HSV-1 gD, to molecules like nectin-2. A much clearer picture underlying this observed difference in the binding properties of the two gDs with their respective receptors will be available after the determination of HSV-2 tr-gD structure.

In this study, we first reported the successful expression and purification of HSV-2 tr-gD protein, which could facilitate soluble-gD-based studies in the future. We also crystallized the protein and obtained high resolution dataset of the crystal. We believe this would be a major step-forward for structural elucidations of features of HSV-2 gD.

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